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TECHNICAL MANUSCRIPT 215

CONTROLLED pH
AND OXIDATION-REDUCTION POTENTIAL
WITH A NEW GLASS
TISSUE CULTURE FERMENTOR

William F. Daniels
David A. Parker
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BIOLOGICAL LABORATORIES
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ACKNOWLEDGMENTS

ABSTRACT

A one-liter fermentor was designed and tested for use as a tissue-
culture vessel. It features a temperature control device; impeller
agitation without the necessity of a shaft seal; and a means for
measuring, recording, and controlling both pH and oxidation-reduction
potential (ORP). Tests have shown the ORP to change fairly rapidly
with impeller speed variations under conditions of a continuous carbon
dioxide - air overlay. Working with strain L mouse fibroblasts (Earle),
cell counts of more than 1.25×10^6 per milliliter without centrifugation
and medium renewal were achieved and cell counts were maintained above
1×10^6 for more than 30 hours. With the vessel studied, pH control
was ±0.05, the ORP control was ±10 millivolts. Controlled environments
for tissue cell metabolic studies are entirely feasible with this
system.

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I. INTRODUCTION

In many laboratories tissue cells are propagated in submerged small stirrer cultures typical of those of Cherry and Hull.[1] McLimans, Giardinello, Davis, Kucera, and Rake[2] have pointed out that this process is difficult to scale up or extrapolate into larger vessels, and they proposed that a 5-liter New Brunswick-type fermentor be used for scale-up studies. Many investigators have acted upon this proposal. Rightsel, McCalpin, and McLean[3] have studied cell growth in 5-, 7½-, and 30-liter fermentors. Cherry and Hull[4] examined growth characteristics of 14 cell strains in 6- and 7-liter fermentors. Hale and Daniels* grew strain L mouse fibroblasts (Earle) in a 40-liter vessel. Perlman and Guiffre[5] have reviewed some of the design considerations for submerged vessels, such as methods of agitation, materials of construction, clumping of cells, air-liquid interface rings of growth, and aeration requirements.

Although the stirrer culture vessel is popular because of its relative simplicity, ease of fabrication, and small size, we feel that it possesses these decided disadvantages: (i) inadequate agitation, resulting in a menstruum that is poorly mixed, poorly aerated, and uneven in temperature; (ii) no provision is made for automatic control and recording of pH values and oxidation-reduction potentials (ORP); and (iii) the process data obtainable are insufficient to permit scaling-up to the standard New Brunswick-type fermentor.

On the basis of the reports by Cooper, Burt, and Wilson[6,7] showing the need for controlled atmospheres and the desirability of control through ORP measurements, and the review of Hongo[8] pointing out the importance of ORP in biological systems; we concluded that ORP control would be desirable and perhaps essential in tissue culture. Cherry and Hull[4] pointed out the importance of rigid control of pH, gas, medium, and other unknown factors in the expansion of suspension culture to an industrial scale. Cater[9] indicated that oxygen tension, ORP, and pH are three process variables that can be physically measured and are of importance in describing the environment of cells in microbiological systems. We concluded from Cater's work that ORP might be a measure of a dynamic equilibrium between an oxygen supply and the metabolic activities of cells represented by a concentration of reduced substances within the system. However, Hongo noted that ORP levels are difficult to measure, and the data even more difficult to analyze. Other workers felt that the dissolved oxygen levels[10] or the oxygen concentration[11] are the most important factors in considering ORP equilibria in systems containing actively growing organisms.

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* Unpublished results.

For these reasons we designed a small fermentor having the following capabilities: (i) its pH and ORP can be controlled and recorded, (ii) it can be aerated at controlled rates and with particular gaseous mixtures, (iii) it can be agitated at desired impeller rates, (iv) it is capable of continuous culture, and (v) its temperature can be sensed and controlled. This report describes the interim design of the fermentor currently used in our tissue culture studies.

II. MATERIALS AND METHODS

A. FERMENTOR

The fermentor parts are shown in Figure 1. The culture vessel (Q) is a 1-liter resin reaction kettle* with a bottom drain and a top (N) with openings for standard-taper 24/40 joints. The center opening contains the impeller assembly (I-M). This assembly consists of a ground-glass housing (I) for a four-poled ceramic magnet** (K) and an impeller shaft; a Teflon sleeve bearing (J); a SFR4 stainless steel ball bearing*** (L) with a 0.25-inch bore, an O.D. of 0.625-inch, and a flange diameter of 0.690-inch; and a stainless steel four-bladed turbine impeller (M) with blades 1/2 by 9/16 inch and an outside diameter of $2\frac{1}{2}$ inches. The impellers were fabricated from 316 stainless steel.

A second opening provided entry for three electrodes: a glass electrode, modified Beckman 19101 redesignated 78304; a silver - silver chloride reference electrode, palladium tip, Beckman 19730, modified by removal of 2 inches within the stem; and a platinum-inlay metallic electrode, Beckman 19001. An exhaust (T) also entered this opening.

Inlet lines (G) for medium, base, and chemical reducing reagents entered through a third opening. On the basis of previous experience we found it necessary to introduce the reagents through small-bore (1-mm) capillary tubes that permitted the reagents to drop directly into the culture. Rubber tubes that ran from medium, base, and chemical reducing reagent reservoirs into the culture vessel were interrupted with ground-glass connections to facilitate breakdown and assembly of the fermentor.

The fourth opening contained the gassing and sampling assembly (A-F). An air-CO_2 mixture or nitrogen was sterilized by passing through two aerosol filters (A and B)**** and then through bacteriological filters of cotton.

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* Fisher Scientific Co., Silver Spring, Maryland.
** General Precision Inc., Aerospace Group, Clifton, N. J.
*** New Hampshire Ball Bearing, Inc., Peterborough, New Hampshire.
**** May be replaced by any suitable high-flow-rate filter.
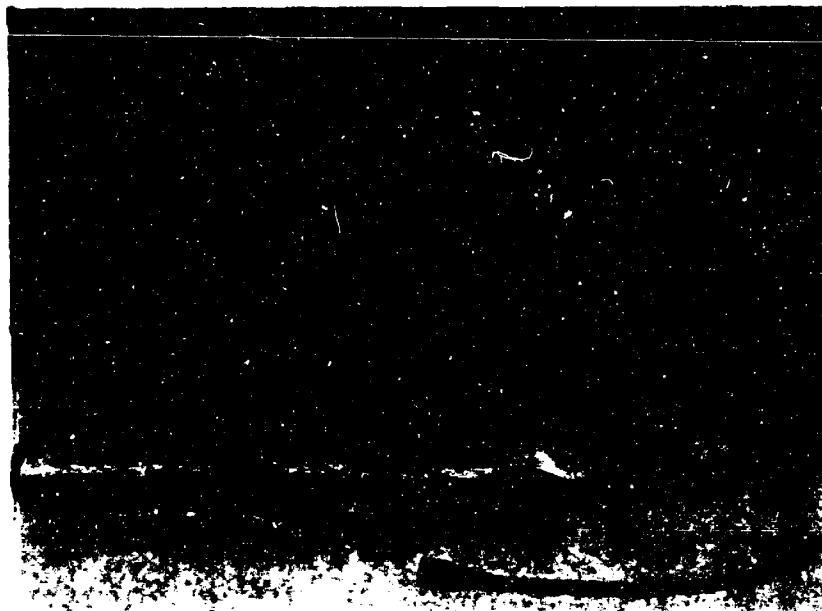
Figure 1. The 1-Liter Fermentor Showing Its Component Parts.
(Neg 18-064-1/AMC-65)

Legend:

A - air filter (two aerosol filter ca..isters, or suitable substitute)
B - nitrogen filter (two aerosol filter canisters, or suitable substitute)
C - bacteriological filter, cotton wool
D - aeration head with thermistor probe
E - entry port
F - sampling port
G - inlet lines for medium, base, and chemical reducing reagents
H - ground glass connectors
I - ground glass housing (24/40 std taper) for magnet and upper shaft
J - Teflon sleeve bearing
K - 4 to 8 pole ceramic magnet
L - stainless steel shaft bearing
M - stainless steel impeller
N - resin kettle top
O - two sheet-rubber gaskets
P - stainless steel electrode holder with rubber stoppers and electrodes
Q - resin reactor kettle. 1000 ml
R - fermentor drain line
S - stainless steel clamp
T - cotton wool filter for air exhaust

A thermistor probe 416* (D) entered with this assembly. An entry port (E) and sampling port (F) are also included in this assembly. A glass tube connected to one of the gas filtering systems and extending into the vessel to the depth of the sampling line permitted sparging. The fermentor was heated with an electrical heating tape** (Figure 2, N) wrapped around the base of the fermentor. The assembled fermentor is shown in Figure 2.

Silastic RTV sealant-adhesive*** was used to seal the rubber stoppers where the electrode leads and various lines entered the fermentor.

B. DRIVE MECHANISM

The agitation system is driven by a sewing machine motor**** (a.c./d.c. 1.2 amp) mounted on an aluminum base plate; the driver pulley is 5/8 inch diameter, and the driven pulley ($3\frac{1}{2}$ inches diameter), is mounted on the shaft with a 4-pole ceramic magnet (Figures 3 and 3a). This drive system, when regulated through a variac on the control panel (Figure 4), will provide suitable speeds. The magnet on the shaft with the driven pulley turns a shaft within the fermentor that has on one end a four- or eight-poled ceramic magnet and the stainless steel impeller on the other. Since one magnet drives the other with a "magnetic cogwheel drive," one can count the exact revolutions of the outside magnet and compute thereby the speed of the inside shaft. At slow speeds the rpm can be determined by counting the number of times a protuberance on the external shaft strikes one's finger within a timed interval; at faster speeds, an arrangement resembling a barber pole is placed on the driver shaft outside the fermentor and a Pioneer Photo-Tach***** Model 12 unit is used to establish its speed.

A further and very considerable advantage of this drive mechanism is the absence of any shaft seal problems.

C. CONTROL PANEL

A control panel (Figure 4) was designed to: (i) control and record pH, (ii) record and control ORP, (iii) delay reactions through the use of suitable timers to prevent overcontrolling, (iv) sense and control temperature, and (v) control the speed of agitation.

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Figure 2. Assembled 1-Liter Fermentor.
(Neg 18-064-2/AML-65)

Legend:

A - shaft top bearing
B - top of thermistor probe
C - air-CO_2 overlay line
D - nitrogen sparge line
E - medium inlet line
F - base inlet line
G - reagent inlet line
H - diaphragm of entry port
I - shaft magnet, 4 or 8 pole ceramic
J - stainless steel clamp
K - sampling line
L - cotton wool filter
M - ground glass connector
N - electrical heating tape
O - filters (aerosol filter canisters or suitable substitute)
P - fermentor drain line
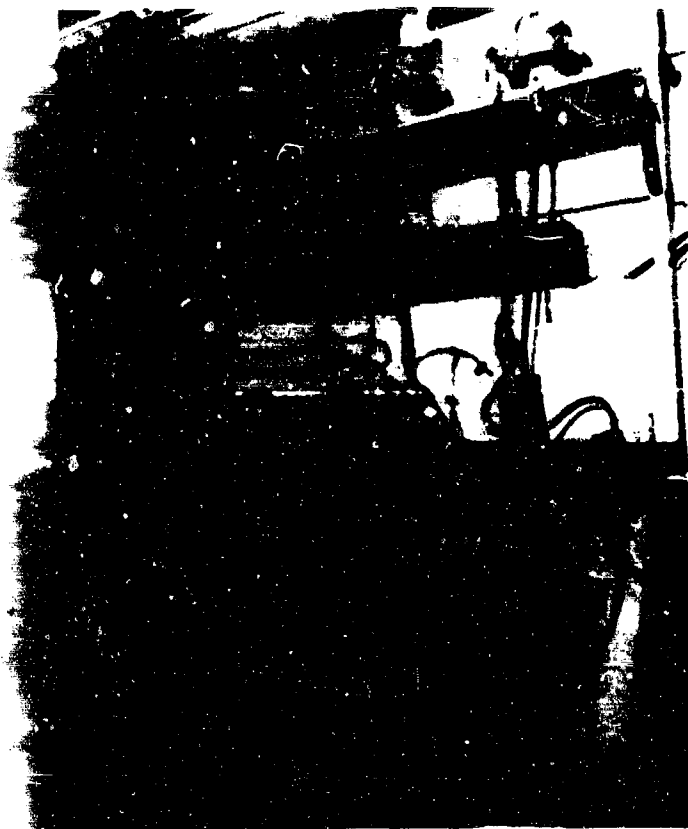
Figure 3. Battery of Three 1-Liter Fermentors with Accessory
Equipment. (Neg 18-064-3/AMC-65)

Legend:

A - medium reservoir
B - base reservoir
C - base measuring burette
D - solenoid valve for base line
E - measuring reservoir for medium additions
F - impeller driving motor assembly
G - stirring motor used for impeller drive
H - assembled fermentor
J - rotameter assembly
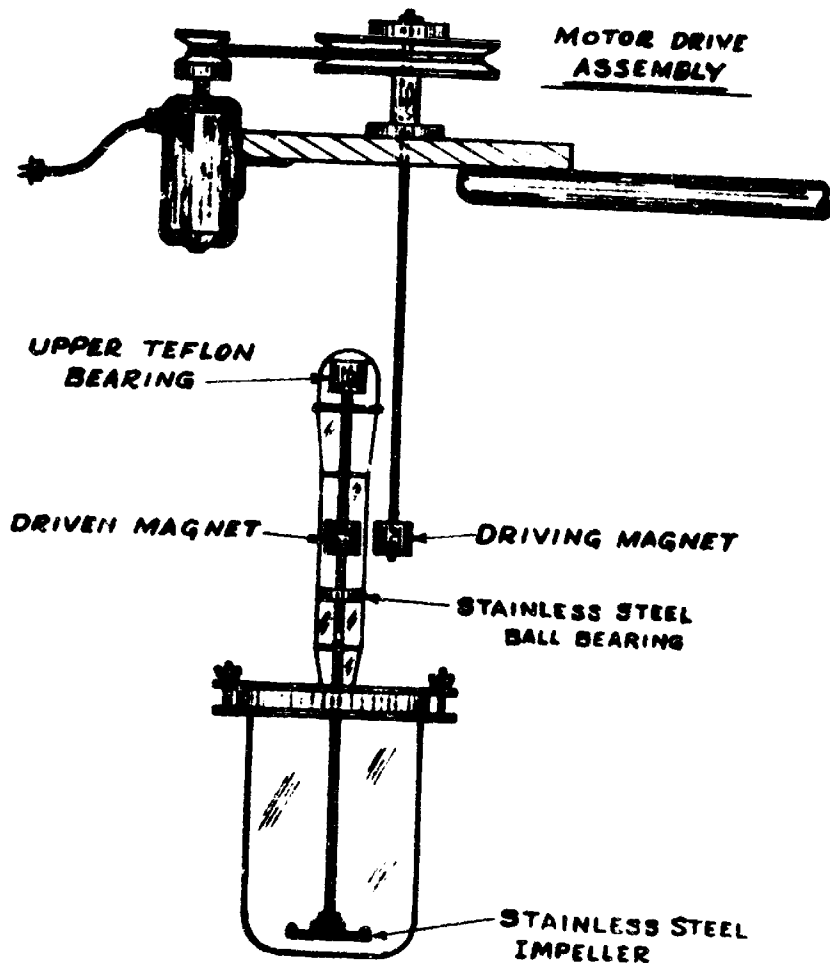
Figure 3a. Simplified View of Fermentor Drive.
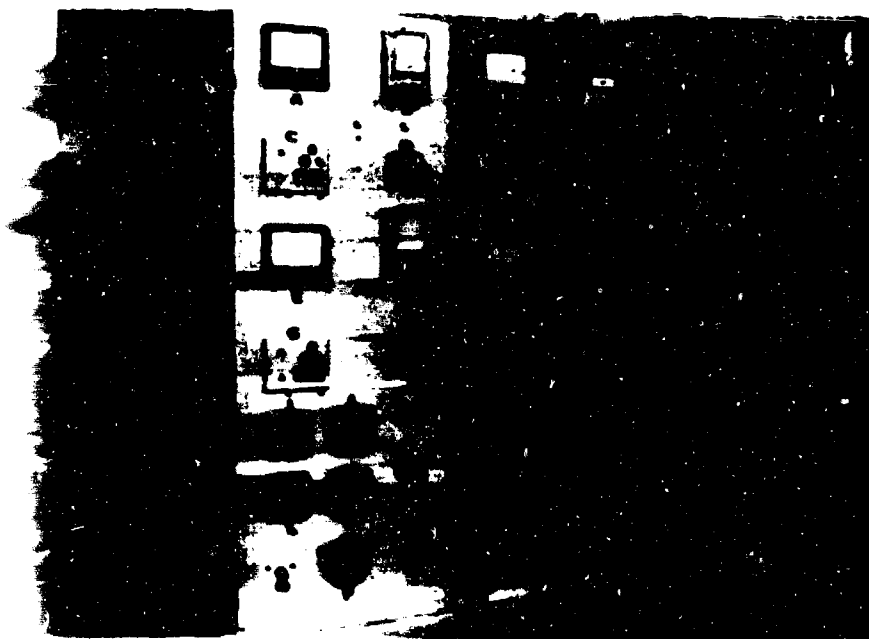
Figure 4. Control Panels for a Battery of Three 1-Liter
Fermentors. (Neg 18-064-4/AMC-65)

Legend:

    A - pH indicator-controller
    B - pH recorder
    C - pH relays
    D - pH asymmetry control
    E - millivolt indicator-controller
    F - millivolt recorder
    G - millivolt relays
    H - millivolt asymmetry control
    I - 0-100% 15-sec timer for pH low point
    J - 0-100% 15-sec timer for pH high point
    K - temperature correction for pH
    L - 0-100% 15-sec timer for millilvolt low point
    M - 0-100% 15-sec timer for millivolt high point
    O - 5-amp fuze
    P - variac
    Q - master switch

The pH and the ORP control devices were identical except that the calibration on the indicator panels differed. Each system consisted of an amplifier (Radiometer PHM-32)[*] with millivolt (mv) and microampere outputs, an indicator-controller (Pantam Messcontactor P x 2 Mil),[**] and d.c. microampere recorder (Rustrak).[***] To record pH, the standard Rustrak 500 - 0 - 500 microampere recorder was used with an appropriate face dial to indicate a pH range of from 2 to 12 pH units. A pH range of from 4 to 10 was used for the indicator-controller, which had a range of 300 - 0 - 300 microamperes. Both the indicator-controller and the Rustrak recorder used for mv measurements had a range of 400 - 0 - 400 mv with an internal range for both of 700 - 0 - 700 microamperes.

The Pantam controller is a relatively inexpensive instrument that indicates the actual value of the circuit after passing the control points. Thus, it does not "peg" and bounce because it is actuated through a system of shadow vanes and photoelectric cells with appropriate relays. This particular circuit employs a separate control box housing that provides sensitivity controls for the two relays (high and low points).

The Rustrak recorder was selected for its low cost and size (3 5/8- by 5 5/8- by 4 1/8-inches). Other commendable features of this current recorder are: (i) 2% full scale accuracy, (ii) 2 seconds maximum response time, (iii) 2% full scale overshoot, and (iv) 31 days recording time with one roll of chart paper and no ink required. In certain instances the recorder was supplied with shunt resistances to provide suitable calibration and operation compatible with the output of the amplifiers.

To prevent overcontrol, ATC Model 304 percentage input controllers[****] were added to time desired corrective actions. For example, this action, when demanded by the indicator-controller for the pH system, limits the time that the valve (Figure 5) of the acid or the base reservoir is open upon receiving a control signal. The maximum and minimum settings are 15 and 2/4 seconds, respectively.

The temperature is controlled through a thermistor probe, a controller[*****] and a heating tape previously mentioned. A small neon "nite-lite" is added to the plug between the heating tape and the controller to indicate on and off cycles at the fermentor proper. Tests showed that the system maintains the desired temperature of 37±0.2 C.

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[*] Radiometer 72, Emdrupvej, Copenhagen NV, Denmark.
[**] Canadian Research Institute, 85 Curlew Drive, Don Mills, Ont., Canada.
[***] Rustrak Inst. Co., 130 Silver St., Manchester, N. H.
[****] Automatic Timing and Controls, Inc., King of Prussia, Pa.
[*****] Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio; Model 63.
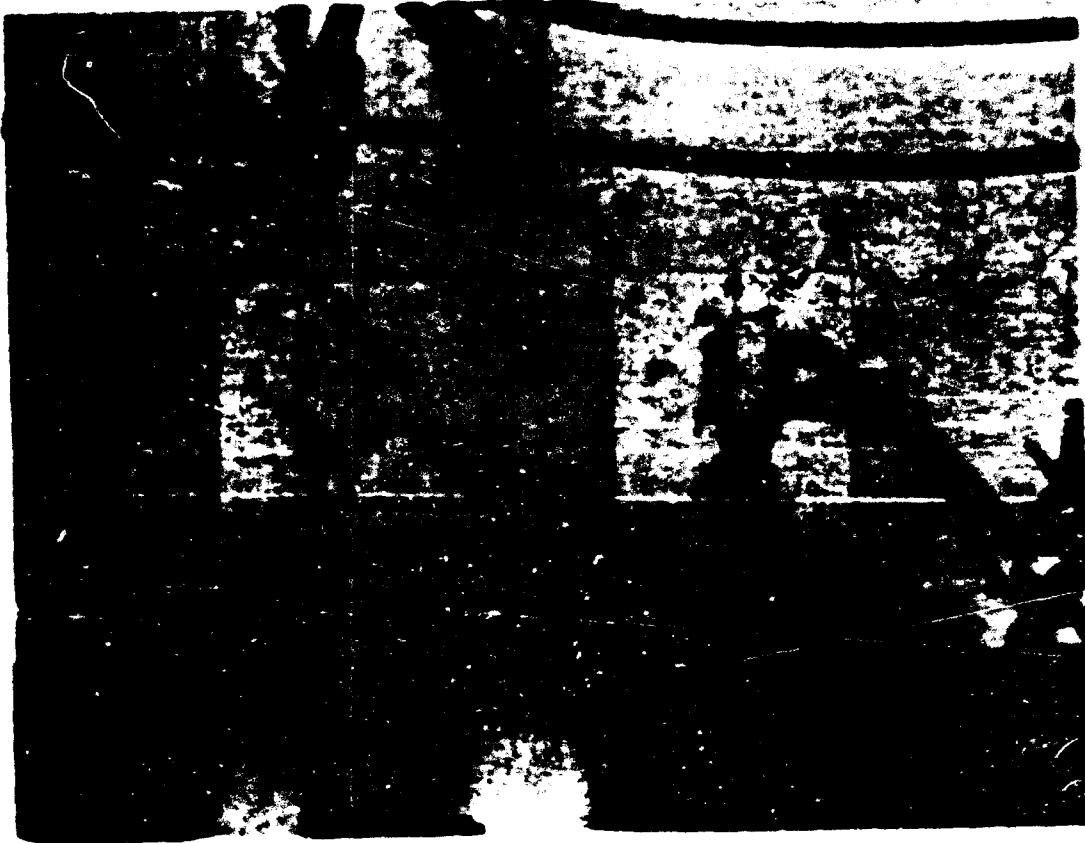
Figure 5. Solenoid Valve Systems.
(Neg 18-064-3/AMC-65)

Legend:

    A - single acting
    B - double acting

D. MEDIUM

Unless otherwise specified, the medium consisted of Eagle's minimum essential medium (double strength) with Earle's balanced salt solution, and 10% bovine serum. This medium was fortified with cysteine, 260 $\mu g/ml$; and ascorbic acid, 50 $\mu g/ml$.

E. CONTROLLING pH AND ORP

The pH of the medium was controlled with sterile solutions of 0.1 N HCl and Na_2CO_3. At very high cell concentrations, when large quantities of reagent may be required to adjust pH, the normality can be changed to 0.5 to prevent excessive dilution and volume buildup.

The ORP was lowered either physically by sparging with N_2; or chemically by adding cysteine, ascorbic acid, sodium thioglycollate, or a mixture of cysteine and ascorbic acid.

Twenty-five-ml burettes with a side arm were used to measure the total base, acid, or other chemicals added on demand to the fermentor. The burettes were filled as needed with previously autoclaved solutions that were stored in 500-ml aspirator bottles. A 4-channel event recorder* showed the time the response took place and its duration. Therefore, if the burette delivers material at a reasonably constant rate, the amount released at any time can be estimated.

A 9-lb Guardian solenoid** was attached to a pinchcock to electrically operate valves that permitted liquids or gases to flow through rubber tubing as desired (Fig. 5). The electrical impulse from the solenoid was used to operate the event recorder to produce a record of the solenoid operation.

F. AERATION OF CULTURE

The gas in the fermentor was controlled by overlaying mixtures of air and CO_2 using the gas mixing apparatus of Daniels and Browning.[12] The flow rate was controlled by a series of rotometers (Fig. 3,J).

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* Rustrak Inst. Co., 130 Silver St., Manchester, N. H.
** Allied Electronics, 100 N. Western Ave., Chicago, Illinois.

G. PROCEDURE

The general procedure for preparing and sterilizing the vessel for tissue cell growth follows:

1. Cleansing of Equipment

The fermentor glassware and all parts were washed and scrubbed with tap water and Micro-Solve* cleansing agent, rinsed three times with tap water, twice with distilled water, and finally rinsed with 10^6-ohm specific resistance water produced by an American Sterilizer** still reserved for production of high-quality distilled water. If the fermentor was not to be used immediately, it was loosely assembled with about 500 ml of the 10^6-ohm water and permitted to soak until needed. This protected the glass electrode from possible dehydration and assured a good rinsing prior to use of the vessel.

2. Assembly

The fermentor as shown in Figures 1 and 2 was assembled, using Dow-Corning stopcock grease on all ground-glass joints as a protective barrier. This lubricant was also used around the stainless steel ball bearing and the Teflon sleeve bearing in the bearing cap because at autoclave temperatures it does not melt and drop into the fermentor.

Openings into the fermentor for wires through stoppers, etc. were sealed with the Silastic sealant-adhesive. Preliminary experiments in our laboratories had shown this material to be relatively nontoxic; cells grew up to the adhesive spread on the bottom of T-60 flasks with no evidence of toxicity.

3. Innoculation

A 250-ml aspirator bottle filled with strain L mouse fibroblast (Earle) cells was used to inoculate the fermentor under aseptic conditions. A needle attached to the aspirator bottle was forced through the entry port (Figure 2,H), which had been sterilized with a 5,000 ppm sodium hypochlorite solution. The cell suspension was allowed to pass into the fermentor by gravity.

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* Micro-Solve Tissue Culture cleaning detergent, manufactured by
  Microbiological Associates, Inc., Bethesda, Md.
** American Sterilizer Co., Erie, Pa.

III. RESULTS AND DISCUSSION

A. EQUIPMENT

1. The Fermentor

Tightness of the apparatus was demonstrated by maintaining a sterile medium within the agitated fermentor for two weeks. Thus, the expedient of using stopcock grease to seal glass joints was of value.

2. Control

a. Response of the pH and ORP systems

In the present design, pH correction is almost instantaneous. Thus, any desired pH could be retained almost indefinitely, or at a desired time the control point shifted and a new pH established. The ORP response generally is slower and depends on the means of correction as to how much time is required before the desired ORP level is established. By adding chemical reducing agents such as a mixture of cysteine and ascorbic acid (26 g/liter and 5 g/liter, respectively), or sodium thioglycollate (112 g/liter), or ascorbic acid (5 g/liter), the time for response to the desired level is about 2 minutes or less. Under the conditions of the experiments to date with either no air overlay or perhaps as much as 5 ml/min, oxidation of the system to the point of requiring an additional corrective action is 1 to 2 hours. The response time for reduction with N_2 sparging is about 15 minutes.

b. Effect of Increasing Impeller Speed

Early in our experiments we noticed that changing impeller speeds produced changes in the ORP registered by the recorder and indicator. We were intrigued by the possibility of controlling the aeration demand of the system via the ORP. The impeller speed was deliberately increased 50 rpm, from 70 to 120 rpm, for 1 minute to determine what effect it would have on the ORP. Figure 6 shows that the ORP rose from a +39 to +62 after 100 minutes, although the speed increase lasted only 1 minute. After an additional 70 minutes, the ORP had dropped to +48, which was still above the initial figure of +39. Approximately 0.191 v should be added to all observed readings of the ORP to obtain the potential relative to the hydrogen electrode.

In attempting to understand just what occurred, we wondered about the actual oxygen transfer rate for this system and the spinner-type vessel with only gas overlays. Table 1 shows the relationships among impeller speeds, volumes, rates of air flow, and sulfite numbers for the
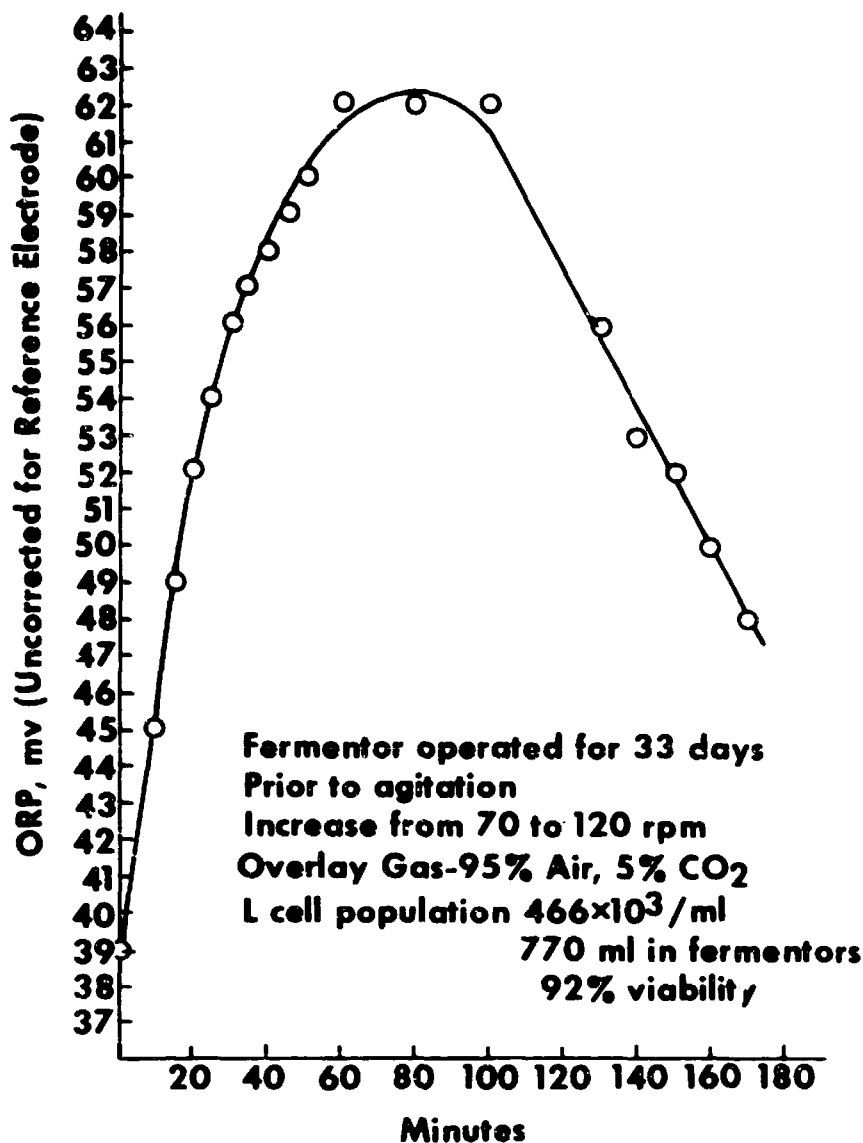
Figure 6. Effect on ORP Level Obtained by Increasing Impeller Speed
50 rpm for One Minute.

fermentor with a gas overlay of 95% air and 5% CO_2. No significant difference exists between the sulfite numbers noted for 5 ml of gas overlay per minute compared with 10 ml. There was no significant difference between the oxygen adsorption rates obtained at 50 and 100 rpm. However, the oxygen adsorption rates did drop significantly as the fluid volume was increased and when the impeller speed was increased to 150 rpm. This phenomenon of decreased adsorption with increased agitation in an unsparged vessel has been reported elsewhere.[13] In the light of these results, the data in Figure 6 become difficult to understand unless we assume that the controlling factor regarding the ORP level established within the system is the rate at which the cells can utilize available dissolved oxygen through their surfaces, and that this rate is enhanced with increased agitation, thus oxidizing the reduced environment. This possibility is being explored, and one can see the possibility of controlling aeration needs of cells and even other microbiological systems by controlling the ORP, since this figure expresses the overall relationship between oxidizing and reducing substances both within and outside the cells.[14]

TABLE 1. SULFITE NUMBERS FOR THE FERMENTOR AT DIFFERENT
AIR OVERLAY RATES AND IMPELLER SPEEDS

Impeller Speed, rpm	Air Flow, ml/min	Sulfite No.[a]/ at Various Fluid Levels in Vessels		
		250 ml	500 ml	750 ml
50	5	0.071	0.034	0.025
100	5	0.073	0.035	0.021
100	10	0.070	0.034	0.022
150	5	0.045	0.027	0.014

a. mM (millimole) O_2/liter/min.

3. Miscellaneous

a. Assembly Time

Initial assembly of equipment, and preparation and sterilization requires much more time (one day per fermentor) than for subsequent runs in which only the fermentor is removed from the lines. In removing a fermentor the lines are clamped and the exposed ends are protected with a gauze pad soaked with 5,000 ppm sodium hypochlorite solution. Using such procedures

a fermentor can be disassembled, cleaned, sterilized, and reassembled in
about 3 hours. Thus, after all valves, air lines, burettes, etc., are in
place, one can change conditions for an experiment, and start another cycle
of three fermentors in about one day; this may be considered a reasonably
efficient operation.

b. Calibration of Electrodes

The ORP system electrodes required special cleaning between runs
and occasional calibration checks to insure accuracy of readings and to insure
that all experiments involving more than one fermentor were initiated under
exactly the same conditions. A sample from the fermentor tested by the
standard laboratory meters for pH and ORP established the initial values
because these electrodes could be exchanged if their accuracy were in doubt,
and a reference sample could be used as a standard also to give a reproducible
value. These findings and special techniques will be presented in
another paper.

B. BIOLOGICAL RESULTS

Results from 30 runs reveal that good growth of L cells can be achieved
within this fermentor, and that accurate pH and ORP control can be attained.
Presently we can achieve pH control within ± 0.05 pH unit and believe that
± 0.1 is acceptable. ORP can be controlled within ± 15 mv with a nitrogen
sparging and aeration/agitation system (95% air and 5% CO_2) or ± 10 mv with
a chemical reduction (such as with ascorbic acid) and aeration/agitation
system. At our present state of knowledge this capability seems acceptable.
Our experience with bacterial systems (unpublished) showed that both pH
and ORP could be balanced much more easily; in fact with Micrococcus
lysodeikticus the air rate could be balanced (sparged air) to give a constant
pH and ORP. We believe that the variation experienced with tissue cells
might arise from either or both of two possibilities:

a) The rate of metabolism is so much slower (generation times of
24 to 48 hours compared with 20 minutes for some bacteria) that the whole
system is sluggish in response; part of this difficulty, too, lies in the
interrelationships among the various constituents of the medium and within
the cell, for example the interrelationships of ascorbic acid, and cysteine -
cystine.[14]

b) Metering small quantities of material exactly as needed is
difficult without unduly complicating the equipment design. We have solved
this problem by using more dilute chemicals, but this approach causes
dilution effects.

Growth of L cells has been equal to or even better in some cases than
generally achieved in spinner cultures not replenished with medium after
centrifugation of the population. Table 2, for example, shows the data
obtained when the fermentor was operated as a spinner culture vessel with
agitation, no pH control, no gas overlay, and no effort to provide ORP
control. Achieving more than 5×10^5 cells per ml without centrifugation
and medium renewal certainly shows that good growth can be achieved in
the vessel, although one might not understand the reasons for failure
in other instances. In one of the earlier experiments, for example, we
noted no growth and checked the air constitution above the liquid surface
to find it to be about 15% CO_2. This, however, does not happen in every
case. Since spinner cultures are generally vented to the air through
cotton-filled glass filters or the like, the regular filling and emptying
of the vessel, through medium additions and removal, supplies necessary
air, even in the absence of a constant air source and pressure such as
presently used.

Table 3 shows the growth of L cells with the electrode system
operating. This experiment was made to determine whether the leakage
of electrolyte through the palladium tip of the reference electrode
would affect growth. The growth is substantially similar to that shown
in Table 2. The experiment was terminated because of contamination picked
up in the medium reservoir.

1. Effect of Adding Cysteine/Ascorbic Acid Mixture to One Run

Table 4 shows observations made during the first 10 days of
a run that lasted 38 days. At the beginning, the growth seemed to falter
and fail so that about 30×10^6 cells, from a 7-day-old Roux bottle, were
added to bolster the population, giving a total of 65×10^6 cells. Here
again the population faltered and the viability dropped from 77 to about
59%. Fifty-two hours after the additional cells were added, automatic
ORP control was begun for reduction, employing cysteine and ascorbic acid
(269 g/liter and 5 g/liter, respectively). The ORP controllers were
arbitrarily set at 0 and +80 mv. Thereafter the cell counts and culture
viabilities steadily increased. At certain points, the automatic control
was not used but the cells reduced the environment themselves, on days
5, 6, 8, 9, and 10.

Table 5 lists the numbers of harvests obtained from the fermentor,
the numbers of generations, and the generation times during the 38-day
period. These harvests were used in other experiments. The yields,
despite the control of pH and ORP, were generally comparable and
only in one instance were they appreciably greater than those previously
obtained from a 4-quart spinner culture. Possible reasons for this
rests in not knowing the right sequence of ORP values, materials to use
for reducing agents, or optimal pH levels. That the growth of cells
might be accelerated by establishing a sequence of ORP values, or whether

TABLE 2. SUBMERGED GROWTH OF EARLE'S STRAIN L CELLS IN THE 1-LITER FERMENTOR OPERATED AS A SPINNER CULTURE WITH EAGLE'S MINIMUM ESSENTIAL MEDIUM

Culture Age		10^3 Viable Cells per ml	10^8 Total Viable Cells [a]	Viability, %	Medium Added, [b] ml	Total Volume, [c] ml	Volume Harvested, [b,d] ml
Days	Hours						
0	0	227	85	95		375	
1	24	170	64	78	20	375	
2	48	318	120	90		376	
2.6	62.4	519	190	95	145	366	
3	72	245	123	75		502	
4	96	522	256	93	145	492	250
4.7	112.7	647	246	97	138	380	
5	120	615	319	92		518	
6	144	688	352	92		512	
7	168	1012	497	94	220	492	365
8	192	575	199	93	144	346	
9	216	416	200	96	145	480	
10	240	435	269	87	158	618	15
11	264	389	293	95		753	
12	288	415	308	93	132	743	490
13	312	301	110	82		365	

a. Expressed as total production, including the cells harvested; 8.9-fold increase obtained over inoculum.

b. Culture harvesting and medium addition performed in that order after sampling for count.

c. This figure represents volume after 1 to 2 samples of 6 to 10 ml were removed for counts.

d. Total amount harvested was 1120 ml, with a total viable count of 710×10^8 cells.

TABLE 3. GROWTH OF EARLE'S STRAIN L CELLS IN THE 1-LITER FERMENTOR, INCLUDING STEAM-STERILIZED pH ELECTRODES WITH EAGLE'S MINIMUM ESSENTIAL MEDIUM

Culture Age		10^3 Viable Cells per ml	10^8 Total Viable Cells	Viability, %	Medium Added, b/ ml	Total Volume, ml
Days	Hours					
0	0	369	107	89		290
1	24	297	83	89		270
2	48	446	121	88	60	261
3	72	447	144	88	90	312
4	96	677	259	87	120	372
5b/	120c/	691	340	87		482

a. Medium added after the sample had been withdrawn for the viable count.
b. The generation time average for the period 1 to 5 days is 47.2 hours.
c. Discarded because of medium contamination.

TABLE 4. OBSERVATIONS OF EARLE'S STRAIN L CELLS GROWN IN EAGLE'S MINIMUM ESSENTIAL MEDIUM IN THE ONE-LITER FERMENTOR.

Day	10^8 Total Viable Cells	Viability, %	Oxidation-Reduction Potential Range,[a]/ millivolts (direction of action)	Remarks
0[b]/	35	55	+170	No control exercised.
1	65	77	+160	No control exercised.
2	55	59	+160	At 52 hr, automatic control[c]/ began between values of 0 to +80 mv.
3	93	81	0 to +125	Automatic control[c]/ between 0 and +80 mv until 76 hr; then automatic control inoperative.
4	110	80	+130	Varied from +120 to 130 mv.
5	175	88	+130 to +80	Dropped from +130 to +80 mv over 24-hr period.
6	244	95	+80 to +40	Dropped from +80 to +40 mv over 24-hr period.
7	254	93	+40 to +100	Cells harvested shortly after 164 hr; aeration probably in excess of need.
7	111	93	+0 to +100	Control between 0 and +100.
8	150	95	+100 to +90	Cells reduce system so that additions of ascorbic acid were not made automatically.
9	242	97	+90 to +80	Reduction by cells.
10	515	98	+80	Reduction by cells balanced by aeration; second cell harvest taken shortly after 236 hr.

a. Uncorrected for Ag-AgCl reference electrode.
b. Culture reseeded with contents of one 7-day Roux bottle culture after three previous days of no growth.
c. Ascorbic acid solution, 5 g/liter, added on demand by controller.

TABLE 5. NUMBER OF GENERATIONS AND MEAN GENERATION TIMES OF HARVESTED
EARLE'S STRAIN L CELLS GROWN SUBMERGED FOR   DAYS IN THE ONE-LITER FERMENTOR

Harvest	Growth Period Between Harvests, hours	10^8 Initial Total Viable Cells	10^8 Final Total Viable Cells	Increased Total Viable Cells, in Generations	Mean Generation Time, hours	Remarks
None	96	14.34	9.3			Inoculum failed to grow; fermentor reseeded.
1	148.6	34.8	319.2	3.197	46.4	
2	20.9	248.3	267.1	0.106	197.2	
3	73	111.1	561.2	2.337	31.2	
4	32.5	135.6	201.2	0.569	57.1	
5	110	179.1	398.6	1.154	95.2	
6	30.4	116.6	22.5			Fermentor drained, flushed, and reseeded; possible toxicity.
7	163.5	179.6	404.8	1.172	139.3	
8	238.5	106.6	752.2	2.819	84.3	
TOTALS	913.4			11.354		Average generation time = 913.4/11.354 = 80.4[a]

a. Average generation time corrected for periods without growth = 69.3 hr.

such levels only serve to poise the cells for growth and then to indicate the progress and speed of growth, still remains to be learned. Even the knowledge of when the logarithmic growth phase has terminated would be of inestimable value in eliminating lag periods in cell production.

One possibly important factor, uncovered at the termination of this run, was the electrolytic decomposition of the impeller, a condition resulting from the use of two different types of stainless steel (306 and 316) in fabricating the impeller. This, of course, with the increased ferric or ferrous ions passing into solution, could have affected the rates of growth.

2. Growth of Cells and Adjustments of ORP via Impeller Speed

The data obtained in the final 169 hours of growth prior to Harvest No. 8 is shown in Tables 6 and 7. During that period the ORP was adjusted by varying the impeller speed ±50 rpm as needed; the volume was held more or less constant, and the medium was fortified with concentrates of Eagle's minimum essential medium and glucose. This resulted in sustaining the cell counts above 1×10^6 cells per ml for 30 hours and probably longer. Our previous experience showed that high cell counts, in the absence of centrifugation of cells and resuspension in fresh medium, dropped rather quickly after occurring. This has been true even though the medium is fortified with Eagle's concentrates, cysteine, and ascorbic acid.

During the 169 hours of this part of the run, the shortest generation time was 14.7 hours and the longest was 84.2 hours. Thus, the average of 45.5 hours (corrected for periods without growth) correlates with that reported in Table 3. The shortest generation time (Table 6) occurred during the period that the cells themselves reduced the environment from +130 mv to +40 mv (Table 7). At 31.9 hours (Tables 6 and 7) the cells were fed with concentrates and left alone during which time the system reduced the potential to +10 mv by 48 hours. The resulting growth showed a generation time of 84.2 hours. The impeller was speeded up to increase the rate of aeration or to raise the potential to +60 at 55.2 hours. The resulting growth showed a generation time of 20.9 hours. The system again dropped to +10 mv. Here the system was fed and raised to +60 mv by increased impeller speed. The cells reduced the system to a +10 with no growth. After this reading at 79.2 hours, the impeller was increased in speed and kept at a faster rate until the ORP rose to +140. The cell numbers increased with a generation time of 77.9 hours. The population then dropped the ORP to a +20 with increased cell growth and a generation time of 24.9 hours. Unfortunately, the experiment was then abandoned, except for counts, for scheduling reasons.

TABLE 6. SUBMERGED GROWTH OF EARLE'S STRAIN L CELLS IN THE ONE-LITER FERMENTOR - FINAL EIGHT DAYS OF GROWTH FOR HARVEST 8 WITH CONSTANT VOLUME

Hours	Vol. of Medium ml	10^3 Viable Cells per ml	10^8 Total Viable Cells	Hours Between Counts	Increased Viable Cells per ml, in Generation, s	Generation Time	Remarks
0	607	371	225				
8.2	767	318	240	8.2	0.131	62.4	Volume includes 160 ml fresh medium and 10 ml 20% glucose
23.9	757	466	348	15.7	0.552	28.5	
31.9	760	658	500	8.0	0.544	14.7	Volume includes 20 ml 20% glucose and 3 ml 200 mM glutamine
48.3	750	754	565	16.42	0.195	84.2	
56.4	764	955	730	8.1	0.386	20.9	Includes 8 ml each of Eagle MEM 100 X concentrates (Microbiol. Ass., Bethesda)
80.4	754	956	720	24.0	0.002		
97.2	744	1110	825	16.8	0.215	77.9	
103.6	755	1291	975	6.4	0.258	24.9	Includes 7 ml of Eagle MEM 100 X sol.
127.3	755	1191	900	23.8			Includes 10 ml 20% glucose
169.2	725	875	635	41.8			
TOTALS				169.2 [a/]	2.283 [a/]		

a. Average generation time = 169.2 hours/2.283 generations = 7.10 hours per generation; average generation time corrected for periods without growth = 103.6 hours/2.83 generations = 45.4 hours per generation.

TABLE 7. OXIDATION-REDUCTION POTENTIAL (ORP) PATTERNS IN THE ONE-LITER FERMENTOR HELD AT CONSTANT VOLUME WITH SUPPLEMENTS OF GLUCOSE, VITAMINS ESSENTIAL AMINO ACIDS, AND GLUTAMINE - FINAL 8 DAYS OF GROWTH FOR HARVEST 8 WITH CONSTANT VOLUME

Hours	10^3 Viable Cells per ml[a]	10^6 Total Viable Cells	Viability, per cent	ORP, millivolts (uncorrected for reference electrode)	Remarks
0	371	225	82	+160	Reduced[b] steadily to +40 at 7.2 hr.
7.2	318	240	93	+40	Fed 160 ml medium, 10 ml 20% glucose; oxidized[c]/ to +130 by increased impeller speed; remained so until 24 hr.
24	466	348	92	+130	Reduced steadily to +40 at 31.2 hr.
31.2	658	500	88	+40	Fed 20 ml 20% glucose, 3 ml 200 mM glutamine; reduced steadily to +10 at 48 hr.
48.0	754	565	88	+10	Oxidized via impeller speed to +60 at 55.2 hr.
55.2	955	730	-	+60	Reduced steadily to +10 at 79.2 hr; fed 8 ml 100 X MEM[d]/ concentrates.
79.2	956	720	83	+10	Oxidized steadily to +140 via impeller at 96 hr.
96.0	1110	825	93	+140	Reduced steadily to +20 at 103.2 hr.
103.2	1291	975	86	+20	Oxidized steadily to +80 at 127 hr; fed 7 ml 100 X MEM concentrates.
127.0	1191	900	90	+80	Oxidized steadily to +150 at 168 hr; fed 10 ml 20% glucose.
168.0	875	635	87	+150	

a. Although the counts are those of Table 6, the hours here correspond to the specific action under remarks and the ORP listed.

b. Reduction was attributable to actively metabolizing cells.

c. Oxidation attributable to either less actively metabolizing cells without increased impeller speed, or to increased impeller speed, or both. Aeration was constant at 5 ml per minute overlay of 5% CO_2 and 95% air. Impeller speed varied between 70 and 120 rpm.

d. Eagle's minimum essential medium.

C. COMMENTS

From the results above one can speculate on the possibility of tailoring aeration needs of the cells or even bacterial systems by use of the ORP measuring and control system. Increasing impeller speeds presents no particularly difficult problems in accomplishing this. Use of nitrogen for reduction is also feasible and has been easily accomplished with this equipment. The exact findings will be presented in another report.

One can only assume that the ORP electrode is an extremely sensitive instrument for measuring aeration, since sulfite numbers (Table 1) show an actual decrease with increased agitation from 100 to 150 rpm with hardly any difference between 50 and 100 rpm. Therefore, the increase in ORP potential caused by increasing impeller speed from 70 to 120 rpm is difficult to understand unless the actual reduction in aeration, as measured by sulfite numbers, is accompanied by increased use of that available by the cells; the increased consumption could arise from better stirring action. That sulfite numbers leave much to be desired for characterizing oxygen transfer rates is common knowledge. Therefore, the findings here may again be an indication of this.

One final comment can be made concerning these experiments compared with those reported by others. These experiments were conducted with no centrifugation of the cells and no replacement of the medium. To feed, the cultures were simply diluted with fresh medium, or Eagle's minimum essential medium concentrates (100 X) were added with or without glucose. Thus, levels of 5×10^6 cells per ml represent fairly high concentrations of cells, particularly when they are remaining in the so-called waste products of metabolism that possibly inhibit growth. The harvests represent withdrawal of cells and medium for other experiments. Dilution of the "heel" (i.e. cells and medium remaining in the fermentor) recharged the vessel.

IV. CONCLUSIONS

From our experience to date we feel these points warranted:

1) A 1-liter fermentor system, with associated equipment, has been designed and found reliable for measuring, recording, and controlling pH levels and oxidation-reduction potentials (ORP). During the growth of L cells, the pH was controlled within ±0.05 pH units and the ORP was controlled within ±10 mv.

2) Tailoring of aeration needs of tissue cell systems and possibly other microbiological systems seems feasible through ORP measurement and control, and is worthy of further study.

3) Controlled environments for tissue cell metabolic studies become entirely feasible with the system described.

The studies reported here in no way are final or representative of what optimal conditions for cell growth might be. Such conditions are the subjects of present studies with the view of eventually being able to increase reliability of submerged growth of cells and consequently increased rates of growth and cell densities.

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13. ABSTRACT

A one-liter fermentor was designed and tested for use as a tissue-culture vessel. It features a temperature control device; impeller agitation without the necessity of a shaft seal; and a means for measuring, recording, and controlling both pH and oxidation-reduction potential (ORP). Tests have shown the ORP to change fairly rapidly with impeller speed variations under conditions of a continuous carbon dioxide - air overlay. Working with strain 1 mouse fibroblasts (Earle), cell counts of more than 1.25×10^5 per milliliter without centrifugation and medium renewal were achieved and cell counts were maintained above 1×10^6 for more than 30 hours. With the vessel studied, pH control was ± 0.05, the ORP control was ± 10 millivolts. Controlled environments for tissue cell metabolic studies are entirely feasible with this system.

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